Granulocyte Colony-Stimulating Factor Versus Placebo in Addition to Penicillin G in a Randomized Blinded Study of Gram-Negative Pneumonia Sepsis: Analysis of Survival and Multisystem Organ Failure

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Sepsis is a common cause of morbidity and mortality. Neutrophils are the major defense against bacterial invasion, and granulocyte colony-stimulating factor (G-CSF) augments both neutrophil number and function. In our study, 160 rabbits were inoculated transtracheally with 0.5 ml of a solution containing 10^4 colony forming units per milliliter of Pasteurella multocida. Twenty-four hours later, chest x-rays and quantitative blood cultures demonstrated pneumonia and bacteremia. Therapy was then begun with penicillin G and either recombinant human G-CSF (rg-CSF; 5 to 8 µg/kg subcutaneously) or placebo every day for 5 days. Arterial blood gases and 23 other parameters of organ function were performed before inoculation and serially thereafter. All rabbits underwent histologic examination of organs at the time of septic death or when sacrificed on day 6. A total of 149 rabbits survived long enough to initiate therapy. A significant increase in leukocytes by day 4 was found in the rG-CSF-treated group. There was a trend towards improved survival in the rG-CSF group (77% v 67%; P = .13, n = 149). Analysis of pretreatment variables revealed sepsis-induced leukopenia (≤2,800/µL) as the only predictor of significantly improved survival with rG-CSF treatment (57% v 39%; P = .04, n = 73). The majority of the survival benefit occurred within the first 24 hours of treatment. This was before the time that a significant difference in mean white blood cell (WBC) count was observed between the study groups, making intravascular leukocytosis an unlikely explanation for the survival advantage in the rG-CSF group. No significant difference in laboratory variables reflecting organ function was demonstrated between the groups. Histologic grading of inflammation (0, normal, to 6, necrosis) in seven organs revealed that the surviving rabbits had mild but statistically significant increased inflammation in the liver, spleen, and noninoculated lung in the rG-CSF versus placebo groups (liver: 2.6 v 1.5, P ≤ .0001; spleen: 3.2 v 2.3, P = .0001; and noninoculated lung: 2.9 v 2.5, P = .04). Administration of rG-CSF, in addition to penicillin G, in immune competent rabbits with gram-negative sepsis complicated by leukopenia significantly improved survival over antibiotics alone. The administration of rG-CSF in early sepsis for a short therapeutic duration was not associated with any clinically evident toxicity. Clinical trials using rG-CSF in septic patients with leukopenia are indicated. This is a US government work. There are no restrictions on its use.

Sepsis is a major cause of morbidity and mortality worldwide. The treatment of any one episode of sepsis may consume an inordinate amount of health care resources due to intensive care unit support, monitoring, end organ damage, and complications due to therapeutic procedures.1 Despite early institution of effective antibiotics and modern supportive intensive care unit measures, a significant percentage of people die of sepsis each year.2 A poor prognostic parameter of survival in sepsis is leukopenia.1,4 Although recent clinical trials have confirmed the benefit of recombinant human granulocyte colony-stimulating factor (rG-CSF) in prevention of chemotherapy-induced neutropenic fevers,5,8 the use of rG-CSF to augment granulocyte number in the immunocompetent individual has not been well studied. Although enhanced bacterial killing and reduced bacterial dissemination could result in improved survival, granulocyte infiltration of tissues with resultant local cellular destruction could also lead to increased organ failure and death.

A survival benefit has been demonstrated with the administration of rG-CSF in animal models of sepsis.9-13 However, most studies of septic immunocompetent hosts have administered rG-CSF before or at the time of bacterial inoculation.9,14 Unfortunately, treatment of all patients with rG-CSF before or at the time of bacteremia would neither be possible nor practical.

This study was designed to test whether the administration of rG-CSF, in addition to antibiotics, would alter morbidity and mortality in animals having gram-negative pneumonia and sepsis. This model was chosen because of the importance of endotoxic shock and pulmonary compromise on survival outcome in humans. Secondary goals were to note whether rG-CSF therapy affected the development of organ failure, and to identify any subgroups that might particularly benefit from rG-CSF treatment. Unlike prior animal studies, this study was designed to simulate clinical sepsis. Using a dosing schedule used in humans, we administered rG-CSF after sepsis was evident.

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MATERIALS AND METHODS

A rabbit model of gram-negative sepsis was used in this study because of the many similarities in metabolic effects that rabbits and humans share in response to endotoxin. On a μg/kg basis, rabbits are less sensitive to the pyrogenic effects of endotoxin than humans; although they do not lose their fever response to repeated exposure. At higher doses of endotoxin, leukopenia, lactic acidosis, hypotension, and death are produced in rabbits, similar to humans with clinically severe gram-negative sepsis. Rabbits and humans have comparable peripheral blood leukocyte responses to human rG-CSF. However, it is unknown if the use of human rG-CSF and endogenous rabbit G-CSF alters other physiologic responses because of molecular differences between human rG-CSF and endogenous rabbit G-CSF.

A total of 160 female, Pasteurella multocida-free, New Zealand white rabbits weighing 2.0 to 2.5 kg each were provided food and water at liberty. The rabbits were anesthetized, and the pretracheal areas were shaved and scrubbed with betadine. A 0.5-cm horizontal incision was made through the cricothyroid membrane, and an 18-gauge, 10-cm catheter was inserted inferiorly into the trachea until resistance was noted. Chest x-rays confirmed the placement of the catheters in the periphery of lung lobes. A 0.5-ml solution of penicillin-sensitive, type III P multocida (a gift from San Diego State University, San Diego, CA) in an exponential growth phase, with a final concentration of 5 × 10^6 colony-forming units (CFU)/ml, was used. Phase 1 of the study determined that the dose of this lethal dose 50% (LD50) when injected transtracheally into the rabbit's trachea was dependent on the administration of 0.3 × 10^-6 U procaine penicillin G administered intramuscularly (IM) every 24 hours, beginning 24 hours after inoculation. Moribidity of the transtracheal procedure was evaluated in phase I by injection of sterile bacterial broth. The clinical and blood parameters outlined below, gross examination at autopsy, and histologic microscopic evaluation after 5 days demonstrated changes limited to postoperative wound healing at the site of the transtracheal incision. No deaths occurred in these rabbits.

For the experimental portion of the study, all investigators were blinded to the type of treatment and laboratory results. Each of the 160 rabbits were treated with procaine penicillin G as above and were randomized additionally to receive human r-G-CSF (Amgen Inc, Thousand Oaks, CA) or placebo each day for 5 days beginning on the day of septic death or after rabbits were sacrificed at 144 hours after inoculation; phase 2 demonstrated no additional deaths after this time period.

Toxicity as a result of sepsis or r-G-CSF administration was assessed over the 144-hour study period. All rabbits were anesthetized for a measurement of rectal temperature and to obtain blood from the femoral artery before inoculation and then at 24, 48, 96, and 144 hours afterward. Each blood analysis included a complete blood cell (CBC) count, electrolytes, liver/renal panel, and an arterial blood gas (ABG) using standard clinical laboratory methods. Hematoxylin and eosin-stained histologic preparations of preassigned organ sites (right and left lung, liver, spleen, gut, kidney, heart) were performed on the day of septic death or after rabbits were sacrificed at 144 hours. The specimens were microscopically graded on degree of inflammation (Table 1) by a single pathologist blinded to the treatment groups. At 24 hours after inoculation, the presence of pneumonitis in each animal was confirmed by chest x-ray (anterior-posterior; Fig 2), and quantitative blood cultures were performed to assess degree of bacteremia.

Survival was analyzed using the Kaplan Meier product limit estimate, and group comparisons were made using Breslow statistics. Serial blood and physical parameters were expressed as the mean ± standard deviation of the treatment groups at each time interval and were compared by the analysis of variance (ANOVA). A P value of less than 0.05 was considered significant. Histologic grading and quantitative blood cultures were evaluated for differences in means by the two-sided, unpaired t-test. Linear regression was performed comparing quantitative blood cultures and white blood cell (WBC) counts and correlated using the Pearson's correlation coefficient.

RESULTS

Before inoculation, 26 blood parameters were measured including CBC count, electrolytes, liver/renal panels, and ABG. These values, as well as weight and temperature, were not different between the 80 rabbits in each treatment group with the exception of a statistically lower creatine phosphokinase in the placebo group than in the r-G-CSF group (895 ± 378 U/L vs 1,098 ± 606, respectively; P = .03). At 24 hours after bacterial inoculation and before any therapy, 11 rabbits died and were excluded from analysis. The 149 remaining rabbits demonstrated clinical illness by lung field consolidation on chest x-rays and significantly increased mean temperatures (preinoculation, 38.8°C ± 0.6°C vs at 24 hours, 40.7°C ± 0.7°C; P < .001), with or without bacteria. Number of cultures positive, quantitative blood cultures, total WBC count, and each of the other parameters above (including creatine phosphokinase) obtained just before therapy revealed no difference between the treatment groups overall or in a subgroup of leukopenic rabbits (<2,800/μL).

WBCs. The mean WBC count for each group showed an initial decrease, followed by a steady increase with time (Fig 3). The rabbits with higher levels of bacteremia were more likely to have leukopenia before treatment (Pearson's correlation coefficient = 4; Fig 4). The WBC counts of all rabbits that lived long enough to receive treatment decreased from a mean preinoculum level of 5,300/μL (range, 2,900 to 10,000) to a pretreatment level of 2,800/μL or less in 49% (n = 73) of the rabbits (mean, 3,800/μL; range, 600 to 19,900), representing sepsis-associated leukopenia. At 96 hours postinoculation, the WBC counts were significantly higher in the treatment group (15,000/μL ± 4,500 v 8,700/μL ± 3,100; P < .001, n = 95) that the control group. At 144 hours postinoculation, this effect was even more pronounced (Fig 3). Figure 5 shows the change in WBC counts for each treatment group further separated into subgroups depending on whether the rabbits were leukopenic or not before treatment at 24 hours. In the placebo group, rabbits that were leukopenic before treatment showed a significant increase in leukocytes at 96 hours compared with those rabbits that were not leukopenic initially (10,300/μL ± 4,600 v 8,200/μL ± 2,300; P = .049). This recovery pattern was not noted in the r-G-CSF-treated subgroups. The magnitude of recovery of the WBC counts at 144 hours was not different between those rabbits that were leukopenic or not before treatment, within each treatment group.
gan to increase within 24 hours of treatment. These trends occurred in both the rG-CSF and placebo groups.

Survival. Overall survival was not significantly different between the rG-CSF and the placebo groups (77% vs 67%, respectively; $P = .13$, $n = 149$; Fig 6). Neither the mean time from inoculation to septic death nor the number of deaths each day differed statistically between the treatment groups. However, in a separate analysis, individual variables were examined to identify a subset of animals that might be responsive to rG-CSF administration. For each pretreatment variable, critical values were determined above or below which all animals either survived or died during the remainder of the study, regardless of whether they received rG-CSF or placebo treatment. These animals were then excluded from analysis, and survival curves were constructed for the remaining animals by treatment group for each variable. Of all the variables, only a leukocyte count $\geq 2,800/\mu L$ was predictive of improved survival with rG-CSF therapy (rG-CSF, 57%; $n = 37$ vs placebo, 39%; $n = 36$; $P = .04$; Fig 7). There was no leukocyte pretreatment value below which all animals died or survived.

Interestingly, the increase in animal survival with the rG-CSF treatment was predominantly within the first 24 hours after treatment. This occurred even though the rG-CSF-treated group did not display a significantly greater WBC count than the placebo group until 72 hours after treatment.

Toxicity. Toxicity was assessed by analyzing the differences in blood parameters of organ function between the treatment groups at each time interval and by histologic evaluation of seven different organ sites. There was no significant difference in organ function between the groups, with the exception of WBC count at 96 and 144 hours. This correlates with prior studies of rabbit and human WBC responses to rG-CSF treatment. No difference in mean organ function at each time period was noted in the group of 149 rabbits overall, in the 73 that were leukopenic ($\leq 2,800/\mu L$) before therapy, or in the 76 not leukopenic (greater than 2,800/\mu L) before therapy. However, both the rG-CSF and placebo treatment groups showed significant parallel changes in nine parameters over time (Fig 3). This toxicity pattern was also duplicated in the leukopenic and nonleukopenic subgroups.

Notably, ABG data were used as an assessment of overall

Table 1. Histologic Grading Criteria for Biopsies of Seven Preassigned Organ Sites

<table>
<thead>
<tr>
<th>Grade</th>
<th>Histologic Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Nonspecific histologic changes (noninflammatory)</td>
</tr>
<tr>
<td>3</td>
<td>Inflammation (mild)</td>
</tr>
<tr>
<td>4</td>
<td>Inflammation (severe)</td>
</tr>
<tr>
<td>5</td>
<td>Inflammation with patchy necrosis</td>
</tr>
<tr>
<td>6</td>
<td>Extensive necrosis</td>
</tr>
</tbody>
</table>
DISCUSSION

Several human studies have shown beneficial results using rG-CSF for the prevention of infectious complication during episodes of chemotherapy-associated leukopenia. Improved survival has also been observed in the treatment of sepsis in animal models when rG-CSF was administered prophylactically. Only one previous study tested the effect of rG-CSF administration after sepsis had been established. In this study, septic peritonitis was induced surgically in rats by cecal ligation and puncture. A single dose of rG-CSF injected 3 hours after cecal ligation and puncture prolonged survival by 75% (n = 12). However, when treatment was begun 6 hours after surgery, survival was not improved. These results suggested that rG-CSF could alter the clinical course of ongoing sepsis even without the addition of antibiotics.

In our study, there was a 10% difference in survival favoring the rG-CSF--treated group, which did not achieve statistical significance. However, in the 73 rabbits with pretreatment leukopenia (WBC count = 2,800/µL), a statistically significant 18% increase in survival occurred in the treatment group (P = .04). None of the pretreatment variables defined a subgroup whose survival was worse with rG-CSF than placebo treatment.

Interestingly, the majority of survival benefit was within the first 24 hours of treatment. This was before the time that a significant difference in mean WBC count was observed between the study groups, making intravascular leukocytosis an unlikely explanation for the survival advantage in the rG-CSF group. We hypothesize that this difference was due to augmentation of neutrophil function or modulation of endogenous cytokines by rG-CSF.

Several studies have demonstrated significantly enhanced neutrophil adherence, membrane depolarization, and superoxide release within 100 minutes of neutrophil exposure to rG-CSF. In vitro, neutrophils exposed to rG-CSF are more adherent to artificial surfaces and have increased expression of C3bi receptors. One might postulate that both early migration of neutrophils out of the vascular space and into sites of bacterial invasion, and enhanced bactericidal capability of phagocytes within the vascular space could hasten bacterial clearance or reduce dissemination and, therefore, lead to increased survival. We observed a significant increase in organ infiltration with neutrophils in the rG-CSF--treated group compared with placebo within the liver, spleen, and noninoculated lung of those rabbits that survived. A difference in inflammation was not observed in the rabbits that did not survive. If these histologic findings are considered to be a specific response to bacterial invasion of one or all of these organ tissues and, therefore, are the cause of the survival advantage with rG-CSF, then migration of neutrophils into the noninoculated lung, liver, or spleen must have occurred within the first 24 hours (time of maximum survival benefit). On the other hand, if this finding is considered to be a nonspecific host response to rG-CSF, then it was not severe enough to shorten survival or affect organ function but may have provided some protection from bacterial spread to these organs. Mechanisms other than neutrophil migration, which are not evident from our data, may have also occurred to account for the early survival benefit.

The protective role of rG-CSF early in sepsis may be independent of neutrophil changes. Elevated levels of tumor necrosis factor (TNF) and interleukin (IL)-1 have been implicated in the pathogenesis of severe septic shock and...
Fig 3. Means and 95% confidence intervals of 10 variables that significantly changed over the 168-hour study period (27 variables total analyzed). No statistical differences were present between the rG-CSF group (---) and placebo group (-----) except for WBC counts at 96 and 144 hours.

multisystem organ failure. Studies of recombinant TNF (rTNF) infusion have produced lactic acidosis, hypotension, and death in animals indistinguishable from the changes produced by lipopolysaccharide (LPS) infusion. Additionally, serum TNF levels have been highly correlated with both severity and mortality in septic shock in humans and organ toxicity after bone marrow transplant. Phase I clinical trials of rTNF administration describe toxic effects including leukopenia, thrombocytopenia, and hypotension. IL-1 is also released in response to LPS and augments the effects of TNF.

Gorgen et al have shown that rG-CSF inhibits LPS-induced production of TNF. They have proposed that rG-CSF may act by feedback inhibition to reduce TNF by a yet unidentified pathway. Bone marrow transplant recipients supported with rG-CSF have had undetectable TNF levels and less organ toxicity when compared with recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF)-treated patients. Attenuation of TNF-induced acute metabolic and hemodynamic changes may affect a survival advantage early in sepsis. The concept that TNF naturally stimulates the cellular release of G-CSF and that this product further inhibits TNF production is appealing and consistent with other models of hormonal regulation. Early antagonism of TNF production by rG-CSF may explain the survival benefit seen in our study.
The rabbits that were leukopenic before treatment had survival rates that were lower on average than those of all rabbits collectively. This correlates with human studies that demonstrate that leukopenia is an indicator of a poor outcome in sepsis. A positive correlation between severity of sepsis and elevated TNF levels has been established in humans. Leukopenia occurs rapidly after TNF injection and may represent one important acute marker of enhanced TNF expression.

Levels of G-CSF, TNF, endotoxin, or neutrophil function were not evaluated in this trial. Measurement of these variables could have contributed to our understanding of the effects of rG-CSF therapy in sepsis. It would have been especially interesting to note if the rabbits that were leukopenic had lower endogenous serum levels of G-CSF initially when compared with those with normal or elevated leukocyte counts. When correlated with survival, low G-CSF levels may have provided a more direct assessment of those
rG-CSF TREATMENT OF SEPSIS: CLINICAL ANALYSIS

Table 2. Histologic Scores (Mean ± SD) of Inoculated and Noninoculated Lungs, Liver, and Spleen in Subgroups Based on Survival Outcome

<table>
<thead>
<tr>
<th>Organ and Treatment</th>
<th>Total</th>
<th>Died</th>
<th>Lived</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rG-CSF</td>
<td>72</td>
<td>14</td>
<td>58</td>
</tr>
<tr>
<td>Placebo</td>
<td>67</td>
<td>20</td>
<td>47</td>
</tr>
<tr>
<td>Noninoculated lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rG-CSF</td>
<td>72</td>
<td>14</td>
<td>58</td>
</tr>
<tr>
<td>Placebo</td>
<td>67</td>
<td>20</td>
<td>47</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rG-CSF</td>
<td>72</td>
<td>14</td>
<td>58</td>
</tr>
<tr>
<td>Placebo</td>
<td>66</td>
<td>19</td>
<td>47</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rG-CSF</td>
<td>69</td>
<td>14</td>
<td>55</td>
</tr>
<tr>
<td>Placebo</td>
<td>66</td>
<td>20</td>
<td>46</td>
</tr>
</tbody>
</table>

*P = .02.
†P = .04.
‡P < .0001.

rabbits that would benefit from exogenous rG-CSF supplementation.

Glucose, temperature, and ABG variations during the study period reflected metabolic changes usual for sepsis. Phlebotomy of 9 mL of blood at each analysis interval (total, 45 mL per rabbit) contributed to the steady decline in hemoglobin. The clinically mild but statistically significant change in platelet number may have been due to suppression of marrow thrombocytopenia by TNF,35,37 as platelet recovery approximated WBC recovery. TNF can also activate the coagulation system by the tissue factor-dependent pathway.44,45 Transient disseminated intravascular coagulation could not be excluded.

A major concern with the use of rG-CSF in sepsis is the possibility of initiating or exacerbating lung dysfunction and acute respiratory distress syndrome (ARDS). To our knowledge, there have been no reported cases of rG-CSF–induced ARDS. Two published case reports describe the use of rG-CSF in ARDS. In the first, one neutropenic patient demonstrated transient decrements in PaO2 of 9 and 14 mmHg at 4 to 6 hours after injections of rG-CSF on 2 consecutive days, although he had a greater decrease in PaO2 of 17 mmHg on the third day, when no r-G-CSF was administered. The patient remained neutropenic, and it was unclear if there was a causal relationship between the PaO2 decrements and treatment with rG-CSF.46 The second report describes resolution of ARDS subsequent to the use of rG-CSF in a neutropenic patient.47

In our study, measurements of lung volume involved with pneumonia were not performed; instead, respiratory compromise was measured by grading a section of each right and left lung for intensity of inflammation and assessing ABG data. Table 2 shows that the inoculated lungs did not differ in mean grade between treatments. The histologic analysis of the noninoculated lung demonstrated more inflammation in the rabbits treated with rG-CSF who survived. This suggests that more rabbits in this group had bilateral pneumonia with an appropriate tissue neutrophilic response, or that rG-CSF induced mild neutrophilic infiltration (grade 3) or nonspecific changes (grade 2) in areas of lung not involved with pneumonia. One study using rats showed that the histologic quantification of neutrophils located in both capillaries and alveolar spaces of both lungs was mildly elevated with 2 days of human rG-CSF treatment.48 If this also occurs in humans, then clinical experience suggests that it is functionally insignificant in the many patients treated with human rG-CSF each day.

The oxygenation curves in Fig 3 suggest that rG-CSF could cause delayed pulmonary toxicity associated with its use in pneumonia-sepsis or a delay in resolution of already established bacterial lung damage. A significant difference in peripheral WBC counts occurred between the groups at 96 hours, inferring a relationship between the increased peripheral WBC count and worsened oxygenation. Conversely, the mean peripheral WBC count was greater and oxygenation better at 144 hours in the rG-CSF group, suggesting that the 5-mmHg difference in PaO2 at 96 hours and the mild inflammatory scores seen in the surviving rabbits were clinically insignificant. Another explanation may be that, early in the course of sepsis, rG-CSF salvaged rabbits that had more extensive morbidity and that would have died without this treatment. These additional, more-compromised rabbits could have caused a reduction in the group’s mean oxygenation value and an increase in the histologic score of the noninoculated lung due to bilateral pneumonia. Therefore, these pulmonary changes may truly reflect a transient rG-CSF–induced pulmonary toxicity that is clinically insignificant, or may represent an improvement in survival of a more-morbid subset due to the addition of rG-CSF in sepsis-associated pneumonia.

Based on our data, treatment with human rG-CSF does not aggravate or protect the rabbit from acute end-organ dysfunction. These findings are in agreement with published data showing that pretreatment with rG-CSF adds no additional pulmonary toxicity in LPS-induced shock.49 Our study did not address therapy prolonged for more than 5 days or the effects of mean leukocyte counts greater than 19,800/μL, which could potentially change the toxicity and survival profiles attained in this trial. Additionally, cardiovascular dynamics were not measured, and alterations in hemodynamics may have affected acute survival without significantly affecting our analyses of organ function. Histologically, the spleens, livers, and noninoculated lungs of surviving rabbits demonstrated mild but statistically significant increased inflammatory scores without ABG or serologic evidence of worsened organ function. Splenic and hepatic neutrophil infiltration and organomegaly and mild lung infiltration have been described with rG-CSF administration in noninfected immunocompetent animals.50,51 In our model of sepsis and our rG-CSF treatment regimen, no clinically significant adverse effects of rG-CSF could be ascertained.

In conclusion, we have shown an early survival benefit with the administration of rG-CSF in addition to antibiotics in sepsis complicated by leukopenia. The administration of rG-CSF in early sepsis for a short therapeutic duration was
not associated with any clinically evident toxicity. Because of the discordance between the period of benefit and the onset of leukocytosis, the mechanism of benefit may not be related to neutrophil number. Early enhancement of neutrophil function or modulation of TNF or other cytokines by rG-CSF may be more important in clinical outcome than was previously suspected. Additional mechanistic studies may be both clinically and scientifically relevant. On the strength of our animal data, we suspect that rG-CSF will be a useful adjuvant therapy for a poor-risk subset of septic patients. Therapeutic trials with rG-CSF given in short duration are, therefore, indicated in humans with sepsis complicated by leukopenia.

REFERENCES


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